

EXHIBIT B2

6d ET-1 Stimulation of 20'FM2030

- $2.5 - 5 \times 10^6$ cells / 150 cm^2 were seeded onto dishes
- cells were grown in P18U x 9 days prior to exp.
- cells repl. c. media 5 phorbol ester for duration of exp. ("NO PMA")
- Media after wash noted, [ET-1] & [ET3] = 10 nM
- 100 nM BQ123 or BQ788 added 1hr before exp. ET addition
- Kersbomycin @ 400 nM ($1/2$ recommended conc.) added 1 day before ET addition \Rightarrow [1] 2°

150 cm^2 Dishes

Protein	
10 nM ET-1	1d
	2d
	3d*
	4d*
	5d
	6d

~~10 ET (6d)~~

~~1d~~
~~2d~~

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150cm² disks

Protein

No ET

6d

(21) 10mM ET-1

1d

2d

3d*

4d*

5d

6d

0.5mM ET-1

6d

1.0mM ET-1

6d

~~10mM ET-1~~ 6d~~10mM ET-1~~ 6d

BQ123/10mM ET-1

4d

BQ123 alone

4d

BQ758/10mM ET-1

4d

BQ758 alone

4d

Kerbomycin/10mM ET-1

1d

Kerbomycin alone

1d

10mM ET-3

6d

RNA

No ET

10mM ET-1

1d

2d

3d

4d

5d

6d

100cm² disks

Protein

No ET

1d

2d

3d*

4d*

5d

6d

7d

8d

9d

10d

11d

12d

13d

14d

15d

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20d

21d

22d

23d

24d

25d

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27d

28d

29d

30d

31d

32d

33d

34d

35d

36d

37d

38d

202

1d NOGT	0.142	1.8	2.78	11.11 \Rightarrow 13.3 (2.4)
1d + ET	0.200	2.2	2.27	9.09 \Rightarrow 10.9 (1.8)
2d NOGT	0.148			7.27 \Rightarrow 8.7 (1.5)
2d + ET	0.234	2.75		20 \Rightarrow 24 (4)
3d NOGT	0.073 (27)	1.0	5.0	22.22 \Rightarrow 26.7 (4.4)
3d + ET	0.126			11.76 \Rightarrow 14.7 (2.4)
4d NOGT	0.082 (27)	0.9	5.56	44.44 \Rightarrow 55.5 (11)
4d + ET	0.176 (27)			18.18 \Rightarrow 24.8 (3.6)
5d NOGT	0.103 (27)	1.7	2.94	
5d + ET	0.170			
6d NOGT	0.109 (27)	0.45	11.11	
6d + ET	0.192 (27)	1.1	4.55	
6d 0.5m ET	0.175 (27)			
6d 1.0m ET	0.249 (27)			
6d ET3	0.285 (27)			
4d BQ123/NOGT	0.126 (77)			
4d BQ78/NOGT	0.163 (27)			
4d BQ123/ET A	0.275 (77)			
4d BQ123/ET B	0.163 (27)			
4d BQ123/ET C	0.235 (27)			
4d BQ123/ET D	0.206 (27)			
1d Herb / NOGT	0.147	1.4		
1d Herb / ET	0.176	1.8		

Cell ① - ECAD cyt 20

$$1 \text{ dNO} - \cancel{6 \text{ dNO}} - 1(\text{A}) - 2(\text{I}) - 3(\text{I}) - 4(\text{I}) - 5(\text{I}) - 6(\text{I})$$

2024 12

Cell - β CAT act 58

$$3x + 27x \cdot 1x$$
$$\frac{1}{x}$$

3dET 5.5/1.4/0.7
4dET 5.6/1.1/6.7
6dET 4.6/0.9/5.5

$$1dNO - 6dNO - 1(t) - 2(t) - 3(t) - 4(t) - 5(t) - 6(t)$$

PM Frichon

0095(17)

2.5X

2.5X

2)	1d -	.115	1.0	2.5	
1.8)	1d +	.228	2.6	0.96	
	2d -	.152	1.5	1.07	
1.5)	2d +	.515	6.7	0.37	
	3d -	.086/.215(2)	0.7/1.2	2.08	
1)	3d +	.267	3.2	0.74	
	4d -	.115/.33(27)	0.9/1.3	2.78/1.92	
1.4)	4d +	.428	5.5	0.45	
	5d -	.165	1.7	3.57-1.47	
4)	5d +	.274	3.3	0.76	
	6d -	.202	2.75	1.11	11.1/2.2/13.3
3.6)	6d +	.296	3.6	0.69	6.9/1.4/8.3
	6d 0.5	.227	2.5	1.0	
	6d 1.0	.270	3.0	0.78	
ET3	4d 0.5	.357	4.4	0.57	
	4d 78/-	.279	3.1	0.74	
	4d 23/+A	.476	6.2	0.40	
	8	.223	2.5	1.0	
	20	.406	5.1	0.49	
	12	.309	3.7	0.68	
	1d Rab 1-	.137	1.3	1.92	
	1d Rab +	.175	1.75	1.43	
	4d 123 /-	.239	2.7	0.93	

Cel ③ ECAD PM

WIND-1(+)-2(+)-3(+)-4(+)-5(+)-6(+)

3X + 27X 1X

Working on

Cel ④ Anty. Prelim.

3X + 27X 1X

4dAb-4d(+)-123(-)-782(-)-123(+)-123(+)-12

Cel ⑤ Titration, ET3

No - 0.5 - 1.0 - 6d - ET3 3X + 27X 1X

Ed.

CIT Factor

0025(1)

202

5X

5000000000

Cel (6) : VbET, Korb

1d 100T

0.000

0.000

0.000

200

200

200

1d 100T

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Results

ECAD:
PM

Duration: ECAD ↓ beginning @ day 1 & almost complete
 shut-off day 5 "Blip" ↑ on day 5 & 6
 decrease well before control sample day 6

* on day 4, ET-1 readout has occurred after midnight i.e.
 put 48hr mark

Antigen/Ab: 123/ET and 788/ET. All labeled "123/ET"
 labeled them A-D. A+C show ↓ ECAD expression:
 will ensure that these are 123/ET and B+D
 show ECAD expression similar to baseline: will ensure
 that there are 788/ET samples & samples return

If above morphs are correct. BQ123 does not inhibit
 ECAD ↓ induced by ET but BQ788 does
 & inhibit ET-induced downregulation of ECAD

Titration time point 6d

ET-1 @ 0.5nM EC ~ 3x ↓ ECAD. Similar ↓ @ 1.0nM
 ~ 6x ↓ NG @ 10nM

ETB 10nM
 ~ 10x ↓ NG of ECAD day 6

Kerbimycin 1d timepoint

- Kerbimycin inhibits ↓ ECAD induced by ET
- why toxic to cells

No ET

↓ in ECAD during course of experiment but
 @ day 1, 4 and 6, ET stimulated samples all c-
 relative ↓ in ECAD expression. Day 2, 3, 5 not tested

110

BCAT cyt

No ETAD in cyt. day 1. Appearance of
ETAD in cyt. day 2-6

2/1/89

100-1

BCAT PM

↑ in mobility, and ↓ protein day 2 and 4.

* ? no Δ mobility day 6 because of late ETAD / no
addition day 4?

BCAT antagonists day 4

mobility shift inhibited by 788 but not 123

BCAT PM BCAT cyt

↑ mobility, day 2, 5, 6

ICAM-1, CD44, NCAM

ET-1 effect

NCAM

Potent ET-1 induced downregulation

~~Conclude~~

Conclusions

- 1) ET-1 decreases E(CAT) protein over 6d time course in late passage (R0 FM2030) melanocytes.
- 2) This effect is mediated by the ETRB subtype
- 3) ET-1 Rx causes the appearance of cytoplasmic E(CAT) beginning day 2
- 4) Herbimycin inhibits ET induced \downarrow in E(CAT) on day 1. Tyr. kinases may be required for same
- 5) ET-1 induced \downarrow E(CAT) is dose responsive and can be noted at concentration as low as 0.5 nM
- 6) ET3, which is selective for ETRB is a more potent \downarrow reg. of E(CAT) than ET-1
- 7) ET-1 induces an increase in motility of melanocytes. β -CAT and a decrease in amt of membrane β -CAT at 48 hrs between 24 & 48 hrs w/ ET-1 stimulation
- 8) ET-1 causes amobility, shift in cytoplasmic β -CAT w/ cell.
- 9) ET-1 is a potent downreg. of MCAM
- 10) ET-1 has no effect on ICAM-1, CD44, and NCA1 expression.

OUGR

BCAT

Future experiments

- 1) Will determine whether $ET-1$ induces β -Tyr (Cytokine)
- 2) Will attempt to see if mobility shift due to dephosphorylation (inhibition of GSK-3)
- 3) Will detach cells from plate and β -Tyr instead of trypan which may be preferable for β -Tyr seen in unstimulated sample.
(i.e. trypanization may cleave β -Tyr, resulting in an increase in expression followed by gradual decrease)